

## Actions of the Protease Inhibitor Phenylmethylsulfonyl Fluoride on Neutrophil Granule Enzyme Secretion and Superoxide Production Induced by fMet-Leu-Phe and Phorbol 12-Myristate-13-Acetate

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**Abstract.** The protease inhibitor, phenylmethylsulfonyl fluoride inhibits granule enzyme release and, above 1 mM, superoxide production from rabbit peritoneal neutrophils induced by the chemotactic peptide, fMet-Leu-Phe. At concentrations below 1 mM, it enhances superoxide production. Superoxide generation stimulated by phorbol 12-myristate-13-acetate is increased by phenylmethylsulfonyl fluoride at all concentrations studied. Phenylmethylsulfonyl fluoride has no effect on the rise in intracellular calcium or the depolarization induced by fMet-Leu-Phe but does decrease the extent of repolarization and abolishes hyperpolarization. It depresses actin polymerization and abolishes cytoplasmic alkalinization caused by fMet-Leu-Phe. The increased phosphorylation induced by phorbol 12-myristate-13-acetate in four of the five proteins studied was not affected by phenylmethylsulfonyl fluoride, but the increased phosphorylation of the fifth, a 21-kD protein was enhanced. We conclude that phenylmethylsulfonyl fluoride acts on inhibitory and enhancing processes or steps induced by fMet-Leu-Phe which are subsequent to or independent of calcium mobilization and protein kinase C activity.

Numerous individuals have participated in delineating at least some of the biochemical steps and sequences involved in the physiologic responses of the neutrophil to stimulation by a variety of soluble and particulate stimuli [reviewed in ref. 17]. With many agonists, the first transduction event, so far identified, is the coupling of receptor stimulation to the activation of phospholipase C by means of a pertussis toxin-sensitive, GTP-binding regulatory protein (G protein) in the neutrophil membrane. The activated phospholipase C hydrolyzes the phosphoinositides to produce two intracellular mediators, inositol 1,4,5-triphosphate (IP<sub>3</sub>) and 1,2-*sn*-diacylglycerol (DAG). IP<sub>3</sub> release Ca<sup>2+</sup> from intracellular stores causing a rise in intracellular Ca<sup>2+</sup> [Ca<sup>2+</sup>]<sub>i</sub>. A variable portion of the increase in intracellular Ca<sup>2+</sup> results from a stimulated influx of Ca<sup>2+</sup> from the extracellular medium. The other intracellular mediator, DAG, is an activator of protein kinase C [15]. Another activator of protein kinase C is phorbol 12-myristate-13-acetate (PMA), a structural analog of DAG [15]. The activation of protein kinase C, and probably

other kinases [10], leads to the stimulus-induced phosphorylation of a number of proteins. The increased activity of protein kinase C is also associated with the activation of the Na<sup>+</sup>/H<sup>+</sup> antiporter; this results in an influx of Na<sup>+</sup> in exchange for the efflux of H<sup>+</sup> with a consequent rise in cytoplasmic pH [17]. In addition to the above, fMet-Leu-Phe, PMA or other stimuli added to a suspension of neutrophils rapidly causes a transient increase in the actin recoverable in the Triton X-100-insoluble fraction or so-called 'cytoskeleton' [18, 29]. Calcium is neither necessary nor sufficient for stimulus-induced actin association [18].

Apart from the above sequence of reactions, a number of investigators have suggested that a protease, more particularly a chymotrypsin-like protease, is involved in stimulus-induced neutrophil functions as diverse as chemotaxis, granule enzyme release, the production of superoxide (O<sub>2</sub>) and phagocytosis [7, 9, 11-13, 16, 23, 26]. The suggestion has been based almost wholly on the effects of protease inhibitors and substrates on the stimulated response [11] and several investigators have questioned this conclusion [1-3, 6,

8, 16a, 21]. Very few of the studies have investigated the mechanism of action of these agents to find out whether they act at one or more of the steps described above [23]. Yet, finding that an inhibitor acted at a single step would support the idea that a protease plays a role in stimulated neutrophil responses. Conversely, finding that it is acting at several steps would suggest that the inhibitor's action is nonspecific. In this work, we have investigated the step or steps at which a widely used, irreversible inhibitor of chymotrypsin and similar proteases, phenylmethylsulfonyl fluoride (PMSF), acts to depress receptor-stimulated granule enzyme secretion and superoxide generation from rabbit peritoneal neutrophils.

## Materials and Methods

Platelet-activating factor (*L*- $\alpha$ -lecithin,  $\beta$ -acetyl, 1-O-alkyl) was obtained from Calbiochem (La Jolla, Calif.) *N*<sup>α</sup>-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe), PMSF, cytochrome C and *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide were obtained from Sigma Chemical Co. (St. Louis, Mo.), PMA from CRC Cancer Biochemicals (Brewster, N.Y.), di-O-C<sub>5(3)</sub> and 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluoresceinacetoxymethylester (BCECF-AM) from Molecular Probes (Junction City, Oreg.) and cytochalasin B from Aldrich Chemical Co. (Milwaukee, Wisc.) Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was a gift from Dr. P. Borgeat (Group de Recherches sur les Leucotriènes, Centre Hospitalier de l'Université de Laval, Qué., Canada). Electrophoresis chemicals were purchased from Biorad Labs (Richmond, Calif.).

Rabbit peritoneal neutrophils were collected 4 or 16 h after the injection of 0.1% shellfish glycogen in sterile isotonic saline and handled as previously described [20]. Hanks' balanced salt solution with no added magnesium was buffered to pH 7.3 with 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES). PMSF-treated cells as well as cells treated with other agents demonstrated >95% viability as measured by the ability to exclude the supravital dye, trypan blue.

The release of lysozyme, *N*-acetyl- $\beta$ -glucosaminidase (NA-Gase) and lactate dehydrogenase (LDH) into the extracellular medium was performed in the presence of 2.5  $\mu$ g/ml cytochalasin B, as described [20]. Lysozyme and  $\beta$ -glucosaminidase release gave similar results so that in most instances only the latter is reported. LDH leakage did not exceed 7% of the total cell content and did not differ between control and treated cells.

A stock solution of PMSF was made in DMSO. Whenever PMSF was used, an equivalent concentration of DMSO was added to the control cells.

Superoxide production was determined at 37°C using the method of Cohen and Chovanec [5]. The O<sub>2</sub><sup>-</sup> was determined as the change in absorbance at 550 nm from baseline measured in a Beckman spectrophotometer Model DU-50. The reaction mixture contained 2.5  $\times$  10<sup>6</sup> cells, 238  $\mu$ M cytochrome C, 2 mM sodium azide, 2 mM CaCl<sub>2</sub> and 2.4 mM MgCl<sub>2</sub>. The reaction was carried out for 2 min. At the end of the specified time, the reaction was stopped by

the addition of 0.5 mM *N*-ethylmaleimide, (NEM). The results reported here refer to endpoint measurements. Generation of O<sub>2</sub><sup>-</sup> was calculated by subtracting the absorbance change in the presence of superoxide dismutase (SOD, 2 mM) from that in its absence and then dividing by the molar extinction coefficient, 21.1  $\times$  10<sup>3</sup> M<sup>-1</sup> cm<sup>-1</sup>.

Quin-2 fluorescence was measured as described [28]. Briefly, suspensions of 10<sup>7</sup>/ml neutrophils were incubated with 10  $\mu$ M quin-2/AM for 30 min at 37°C, washed twice in indicator-free Hanks' buffer containing 1.6 mM calcium and resuspended in the same buffer to the same cell concentration and equilibrated at 37°C for 10 min. Cell fluorescence was measured in an SLM 8000 fluorescence spectrophotometer. The excitation and emission wavelengths were 390 and 520 nm, respectively.

Cytoskeletal actin was isolated and measured as previously described [19]. Briefly, 0.5-ml aliquots of 5  $\times$  10<sup>6</sup> cells were distributed into various sets of microcentrifuge tubes. The reactions were started by the addition of the stimuli and stopped by adding cold 1% Triton X-100 stock solution. The cytoskeletal proteins were electrophoresed through 10% polyacrylamide slab gels, stained with Coomassie blue and the band corresponding to actin measured by densitometry.

Changes in the intracellular pH of the neutrophils were determined by the procedure described by Weissman et al. [27]. Briefly BCECF-loaded neutrophils at a cell density of 1  $\times$  10<sup>7</sup>/ml were incubated for 5 min in the SLM 8000 spectrofluorimeter with excitation and emission wavelengths set at 500 and 530 nm, respectively. The cell was then stimulated with fMet-Leu-Phe.

Transmembrane potentials were determined by means of the potential sensitive dye, di-O-C<sub>5(3)</sub> [4], as previously described [14]. In brief, neutrophils suspended at a final concentration of 10<sup>6</sup> cells/ml were incubated with a final concentration of 5  $\times$  10<sup>-7</sup> M dye for about 2 min in the SLM 8000 spectrofluorimeter until steady-state fluorescence was attained. The various stimuli were added and fluorescence was measured. The excitation and emission wavelengths were 460 and 510 nm, respectively.

## Results

### Degranulation

PMSF inhibited granule enzyme release induced by fMet-Leu-Phe (fig. 1). The extent of the inhibition was almost completely independent of the strength of the stimulus (fig. 1). The inhibition was zero at 0.5 mM PMSF and began to attain maximum levels at 2.0 mM (fig. 2).

### Superoxide Generation

Unlike the situation seen with degranulation, concentrations of PMSF of less than 1.0 mM reproducibly enhanced O<sub>2</sub><sup>-</sup> generation (fig. 3), although PMSF at 1.0 mM or above gave a concentration-dependent inhibition similar to that seen with granule enzyme secretion. The inhibition was reversed by washing the cells (fig. 4). In contrast to the findings with cells stim-

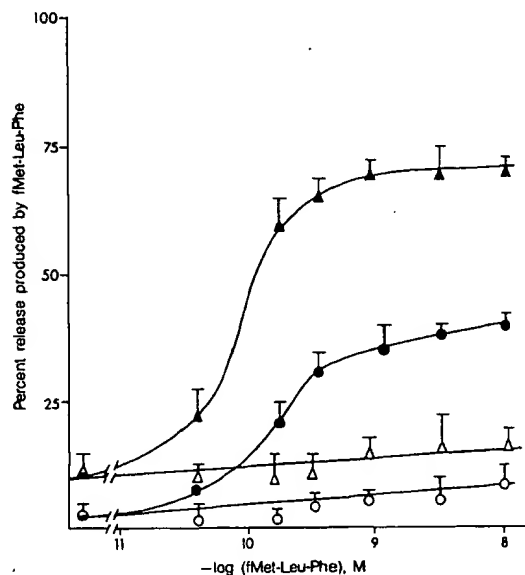


Fig. 1. Inhibition by 2 mM phenylmethylsulfonyl fluoride (PMSF) of the degranulation of rabbit neutrophils induced by varying concentrations of fMet-Leu-Phe and 2.5  $\mu\text{g}/\text{ml}$  of cytochalasin B. PMSF was preincubated with the cells at 37°C for 5 min. Each point is the mean of at least 3 separate experiments  $\pm$  standard error. ● = Glucosaminidase release by control cells; ○ = glucosaminidase release from neutrophils treated with PMSF; ▲ = lysozyme release by control cells, △ = lysozyme release by PMSF-treated cells. The cell suspension contains  $10^7$  cells/ml.

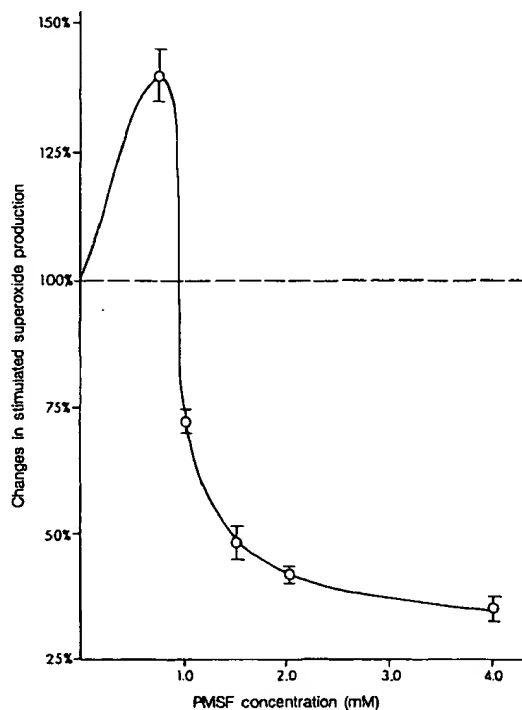


Fig. 3. Effect of varying concentrations of phenylmethylsulfonyl fluoride (PMSF) on the amount of superoxide ( $\text{O}_2^-$ ) generated from rabbit neutrophils following stimulation with  $5 \times 10^{-9}$  M fMet-Leu-Phe and 2.5  $\mu\text{g}/\text{ml}$  of cytochalasin. The experimental conditions are the same as described in 'Materials and Methods'. The PMSF was preincubated with the cells at 37°C for 5 min. Each point is the mean of at least 3 experiments  $\pm$  standard error. The cell suspension contains  $10^7$  cells/ml.

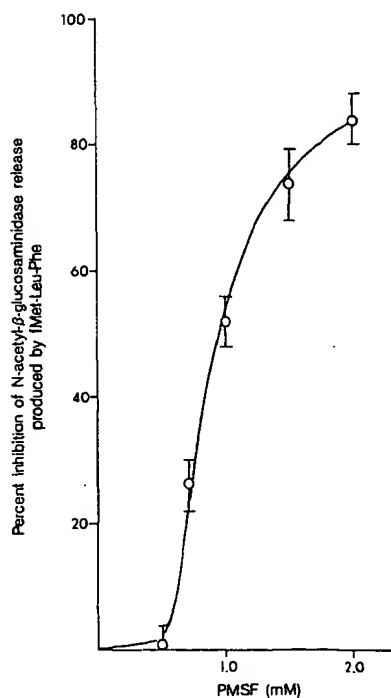


Fig. 2. Inhibition by varying concentrations of phenylmethylsulfonyl fluoride (PMSF) of the degranulation of rabbit neutrophils induced by  $10^{-9}$  M fMet-Leu-Phe and 2.5  $\mu\text{g}/\text{ml}$  cytochalasin B. The experimental conditions were the same as for figure 1. Each point is the mean of at least 3 experiments  $\pm$  standard error.

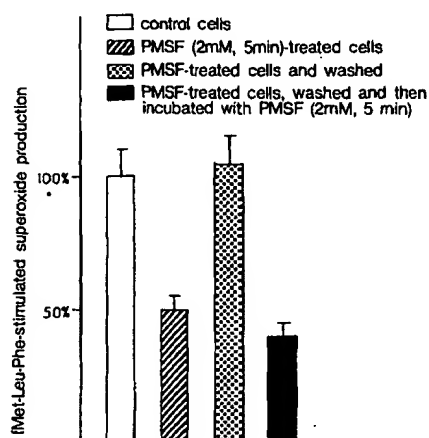


Fig. 4. Reversibility of the inhibition by 2 mM phenylmethylsulfonyl fluoride (PMSF) of the enhancement of superoxide produced in response to  $5 \times 10^{-9}$  M fMet-Leu-Phe and 2.5  $\mu$ g/ml cytochalasin B. The experimental conditions are described in 'Materials and Methods'. The PMSF was preincubated with the cells at 37°C for 5 min. The bars represent the mean of 3 experiments  $\pm$  standard error.

ulated with fMet-Leu-Phe, the extent of  $O_2^-$  generated in response to PMA was uniformly enhanced even by high concentrations of PMSF (table 1).

#### Cytoskeletal Actin

As is evident from table 2, 2 mM PMSF inhibited the increase of cytoskeletal actin induced by either fMet-Leu-Phe or PMA.

#### Cytoplasmic Free Calcium

Treating the neutrophil with 2 mM PMSF did not interfere with the ability of fMet-Leu-Phe to increase intracellular calcium, as measured by the fluorescence of quin-2. Concentrations of PMSF even higher than 2 mM had no effect on the rise in intracellular calcium (not shown). PMSF was also unable to inhibit the increase of intracellular calcium caused by stimulation with  $LTB_4$  or platelet-activating factor (table 3).

#### Membrane Potential

Another early response to the stimulation of neutrophils by a number of agonists, including fMet-Leu-Phe, are changes in membrane potential. These changes are manifest as a rapid depolarization (decrease in fluorescence; fig. 5) followed by a hyperpolarization (recovery to baseline and even above; fig. 5). As is evident from figure 5, PMSF did not affect the depolarization phase although it decreased the subsequent repolarization.

Table 1. Effect of PMSF on superoxide generation in rabbit neutrophils produced by PMA

PMA concentration ng/ml	Superoxide generation, nmol/ $10^7$ cells, in 5 min		
	control	+ PMSF (2mM)	
50	$19.9 \pm 2$ (n = 4)	$26 \pm 4$ (n = 3)	$p \leq 0.05$
100	$63 \pm 10$ (n = 5)	$110 \pm 20$ (n = 4)	$p \leq 0.001$

The numbers in parentheses refer to the number of experiments. The cell suspension contains  $10^7$  cells/ml.

Table 2. Basal and stimulated cytoskeletal actin in control and PMSF-treated rabbit neutrophils

Condition	Cytoskeletal actin, relative to control		
	control	PMSF-treated	
No addition	1.0	$1.12 \pm 0.05$	NS
+ fMet-Leu-Phe* (1 nM)	$2.10 \pm 0.2$	$1.30 \pm 0.10$	$p \leq 0.001$
+ PMA* (100 ng/ml)	$1.50 \pm 0.15$	$1.10 \pm 0.08$	$p \leq 0.01$

Cells were treated with 2 mM PMSF for 5 min. The cell suspension contains  $10^7$  cells/ml. NS = Not significant. Each value represents the mean  $\pm$  SEM of at least 3 separate experiments.

\* Cells were stimulated with fMet-Leu-Phe for 20 s and with PMA for 3 min.

Table 3. Effect of PMSF on the rise in intracellular concentrations of free calcium in human neutrophils produced by various stimuli

Stimulus	Increase in cytoplasmic-free calcium, nM	
	control	+ PMSF (2 mM, 5 min)
fMet-Leu-Phe, 10 nM	$468 \pm 50$	$423 \pm 69$
Leukotriene $B_4$ , 10 nM	$633 \pm 37$	$578 \pm 48$
Platelet-activating factor, 10 nM	$616 \pm 68$	$550 \pm 29$

Each value represents the mean  $\pm$  SEM of 4 experiments. The cell suspension contains  $10^7$  cells/ml. The differences are not significant.

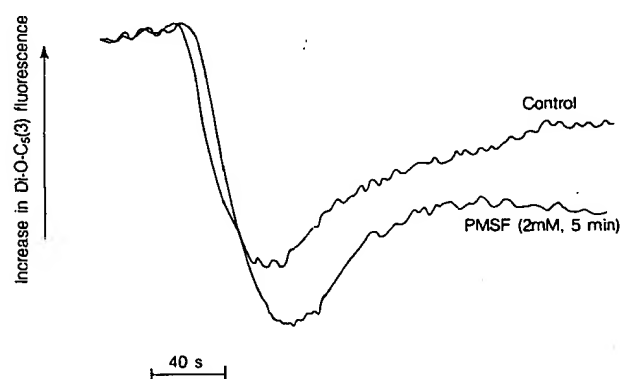


Fig. 5. The effect of 2 mM phenylmethylsulfonyl fluoride (PMSF) on the changes in fluorescence of di-O-C<sub>3</sub>-loaded rabbit neutrophils ( $2 \times 10^6$  cells/ml) stimulated with  $10^{-9}$  M fMet-Leu-Phe. The neutrophil suspensions were loaded with di-O-C<sub>3</sub> as described in 'Materials and Methods'. The cells were preincubated with PMSF at 37°C for 5 min before the addition of fMet-Leu-Phe (arrow). The tracings are from 1 experiment and are typical of 3 such experiments.

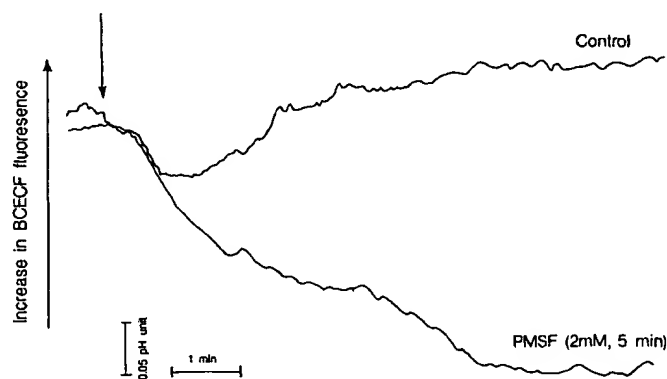


Fig. 6. The effect of 2 mM phenylmethylsulfonyl fluoride (PMSF) on the change in fluorescence of BCECF loaded cells induced by  $5 \times 10^{-8}$  M fMet-Leu-Phe. The neutrophil suspensions ( $10^7$  cells/ml) were loaded with BCECF as described in 'Materials and Methods'. The cells were preincubated with PMSF at 37°C for 5 min before the addition of fMet-Leu-Phe (arrow). The tracings are from 1 experiment and are typical of 3 such experiments.

Table 4. Phosphorylation of several proteins by PMA in control and PMSF-treated rabbit neutrophils

Protein	Increased radiolabeled <sup>32</sup> P-incorporation relative to control			
	control	+ PMA	+ PMSF	PMSF + PMA
21 kD	1.0	1.40 ± 0.2	0.86 ± 0.05	3.0 ± 0.20
35 kD	1.0	1.41 ± 0.1	1.2 ± 0.03	1.66 ± 0.15
46 kD	1.0	1.60 ± 0.15	0.95 ± 0.10	1.50 ± 0.08
56 kD	1.0	1.90 ± 0.12	1.12 ± 0.11	2.04 ± 0.12
72 kD	1.0	1.65 ± 0.15	1.17 ± 0.08	1.60 ± 0.09

Cells ( $10^7$  cells/ml) were incubated with 2 mM PMSF for 3 min, then stimulated with PMA for 5 min. Each value represents the mean ± SEM of at least 3 separate experiments.

#### Changes in Cell pH

A number of stimuli induce an initial decrease in cell pH followed by an increase. As shown in figure 6, PMSF does not affect the initial decrease in pH induced by fMet-Leu-Phe (fall in fluorescence) but entirely prevents the subsequent cell alkalinization (increase in fluorescence).

#### Protein Phosphorylation

PMA increases the phosphorylation of at least nine proteins [29], five of them (table 4) to a readily measurable extent. PMSF does not affect the unstimu-

lated level of phosphorylation of any of the five proteins nor the increased level of phosphorylation induced by PMA of four of these five. However, it enhanced fivefold or more the PMA-induced increase in phosphorylation of the 21-kD protein.

#### Discussion

In this investigation, we have shown that preincubation of phenylmethylsulfonyl fluoride (PMSF) with rabbit peritoneal neutrophils for 5 min decreases granule enzyme release induced by fMet-Leu-Phe in a concentration-dependent fashion. The concentrations required are relatively high, the threshold being  $> 500 \mu\text{M}$ . The effect of PMSF on  $\text{O}_2^-$  production caused by the same stimulus is biphasic; at concentrations less than 1 mM, PMSF causes an enhancement, whereas, at 1.0 mM or greater a dose-dependent inhibition is evident. Kitagawa et al. [12] found that PMSF, at approximately the same concentrations as used here, inhibited  $\text{O}_2^-$  production from human neutrophils when cytochalasin E or Con A was the stimulus they did not observe enhancement. Their results were similar to those of Rao and Castranova [16a]. It is not known whether the lack of enhancement found by these authors is due to the difference in the stimulus or in the species of cells. Whatever the basis, with rabbit cells stimulated by fMet-Leu-Phe, PMSF ap-

pears to act on two opposing processes or at two different steps to affect  $O_2^-$  production, an enhancing process or step evident at lower concentrations of PMSF and an inhibitory one seen at higher concentrations. However, with granule enzyme release induced by the same ligand, only the inhibitory process is evident. This might explain why concentrations of PMSF required to inhibit  $O_2^-$  generation caused by fMet-Leu-Phe are somewhat greater than those required to inhibit granule enzyme secretion to the corresponding extent. In contrast when PMA is the stimulus, PMSF, no matter how high the concentration, only enhances  $O_2^-$  generation. Here, it appears that PMSF acts only at the enhancing step or process.

Even at concentrations above 2 mM, PMSF has little or no effect on the rise in intracellular  $Ca^{2+}$  induced by fMet-Leu-Phe despite the fact that 2 mM PMSF causes an 80% or greater inhibition of enzyme secretion and a 60% or greater inhibition of  $O_2^-$  production. The rise in intracellular  $Ca^{2+}$  induced by  $LTB_4$  or PAF also is not affected by PMSF. The lack of effect on intracellular  $Ca^{2+}$  indicates that PMSF acts either after the rise in  $Ca^{2+}$  or on a step in a biochemical sequence parallel to the  $Ca^{2+}$  step. The result also implies that PMSF does not interfere with the steps leading up to the rise in  $Ca^{2+}$ , that is, the binding of fMet-Leu-Phe to its receptor, the coupling of receptor and G protein with activation of phospholipase C and the production and activity of inositol 1,4,5-trisphosphate.

The inhibition of fMet-Leu-Phe-induced actin polymerization found here with rabbit neutrophils agrees with what has been reported for human neutrophils [16a]. Evidence has been presented that a rise in intracellular  $Ca^{2+}$  is neither necessary nor sufficient to induce actin polymerization in neutrophils following stimulation with fMet-Leu-Phe [18]. Thus, the finding that PMSF inhibits actin polymerization induced by the chemotactic peptide is compatible with the conclusion that PMSF does not interfere with the rise of  $Ca^{2+}$  and suggests that it acts independent of the subsequent step or steps contingent on the rise in  $Ca^{2+}$ .

The inability of PMSF to affect the depolarization phase of the change in membrane potential induced by fMet-Leu-Phe also indicates that the inhibitor is not acting on the earliest steps in the biochemical sequence(s) induced by fMet-Leu-Phe stimulation.

There is evidence that PMA induces  $O_2^-$  generation in neutrophils by translocating protein kinase C to the plasma membrane and activating it [17]. The fact that

PMSF does not inhibit but rather stimulates  $O_2^-$  production induced by PMA suggests that PMSF does not interfere with either process. Support for this conclusion is given by the results of the measurement of the effects of PMSF on the protein phosphorylation presumably caused by an increase in the activity of protein kinase C induced by PMA [17]. PMSF did not affect the PMA-induced increase in phosphorylation of four of the five proteins studied. The lack of effect on these proteins also suggests that PMSF affects neither the activation nor the resultant activity of protein kinase C. In view of this, one possible explanation for the ability of PMSF to greatly enhance the phosphorylation induced by PMA of the 21-kD protein is that PMSF inhibits a protein phosphatase more or less specific for that protein. Whatever the explanation, PMSF enhances both the increase in the phosphorylation of the 21-kD protein and the increase in  $O_2^-$  induced by PMA. This suggests the possibility that phosphorylation of the 21-kD protein is involved in  $O_2^-$  production. Although the myosin light chain has a molecular weight of 21 kD, whether the 21-kD band demonstrable by one-dimensional gel electrophoresis represents a single protein is not known.

The view that PMSF does not directly affect protein kinase C activity implies that PMSF inhibits granule enzyme secretion and  $O_2^-$  production induced by fMet-Leu-Phe at a step or steps occurring after protein kinase C involvement or in a sequence parallel to the one leading to protein kinase C involvement.

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